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Published in:
Archives of Microbiology

DOI:
10.1007/BF00409104

Arch. Microbiol. 120, 167–175 (1979)

Archives of
Microbiology

Characterization of Peroxisomes in Glucose-Grown *Hansenula polymorpha* and Their Development after the Transfer of Cells into Methanol-containing Media

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Abstract. Cells of *Hansenula polymorpha* growing exponentially on glucose generally contained a single peroxisome of small dimension, irregular in shape and located in close proximity to the cell wall. Crystalline inclusions in the peroxisomal matrix were not observed. Associations of the organelles with one or more strands of endoplasmic reticulum were evident. In stationary phase cells the size of the peroxisomes had increased considerably. They were more cubical in form and showed a partly or completely crystalline matrix.

After the transfer of cells growing exponentially on glucose into media containing methanol, large peroxisomes with a partly crystalline matrix developed in the cells within 6 h. These organelles originated from the small peroxisomes in the glucose-grown cells. De novo synthesis of peroxisomes was not observed. Prolonged cultivation in the presence of methanol resulted in a gradual increase in the number of peroxisomes by means of separation of small peroxisomes from mature organelles. During growth of peroxisomes associations with the endoplasmic reticulum remained evident.

The increase in volume density of peroxisomes in stationary phase cells grown on glucose and in methanol-grown cells was accompanied by the synthesis of the peroxisomal enzymes alcohol oxidase and catalase. Cytochemical staining techniques revealed that alcohol oxidase activity was only detected when the peroxisomes contained a crystalloid inclusion. Since in peroxisomes of an alcohol oxidase-negative mutant of *Hansenula polymorpha* crystalline inclusions were never detected, it is concluded that the development of crystalloids inside peroxisomes is due to the accumulation of alcohol oxidase in these organelles.

Key words: Peroxisome – Biogenesis – Yeast – *Hansenula polymorpha* – Cytochemical staining – Methanol

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Growth of yeasts on methanol as the sole source of carbon and energy is characteristically associated with the presence of clusters of peroxisomes, whose number and substructure are dependent on cultivation conditions of the cells (Van Dijken et al., 1975a; Fukui et al., 1975a; Sahm et al., 1975; Veenhuis et al., 1978). It has been shown that the organelles are involved in methanol metabolism and contain the two enzymes which catalyze the oxidation of methanol to formaldehyde, namely alcohol oxidase and catalase (Van Dijken et al., 1975b; Fukui et al., 1975b; Roggenkamp et al., 1975; Veenhuis et al., 1976). During growth on glucose peroxisomes are absent or hardly detectable (Van Dijken et al., 1975a; Parish, 1975; Sahm, 1977).

In contrast to their well-described structure and function, surprisingly little is known about the biogenesis of peroxisomes so abundantly present in methanol-grown yeast cells. Two contradictory hypotheses have been put forward with respect to the origin of these organelles, neither of which has been sufficiently substantiated. Tsubouchi et al. (1976) have described de novo synthesis of peroxisomes in cells of *Candida* sp. N16 during the lag after the transfer of cells from glucose- to methanol-containing media. On the other hand, Tanaka et al. (1976) assumed the presence of a preexisting organelle in glucose-grown cells, from which the peroxisomes, after the transfer of cells to methanol, originated by a process of growth and division.

In this papar we present ultrastructural and biochemical evidence for the presence of a small peroxisome in glucose-grown cells of *Hansenula polymorpha* which differs structurally and functionally from peroxisomes in methanol-grown cells. It is shown that this organelle is converted – during the lag after the transfer of glucose-grown cells to methanol-containing media – to a peroxisome, typical of those contained in yeasts grown on methanol and that multiplication of this organelle occurs by division.

0302-8933/79/0120/0167/$01.80

Materials and Methods

Microorganism and Cultivation

Hansenula polymorpha de Morais et Maya CBS 4732 was used in all experiments. The organism was grown in 500 ml shake flasks at 37° C in the medium described previously (Veenhuis et al., 1978).

Before transfer to media with methanol as the sole carbon- and energy source, the cells were precultured in media containing 0.25% glucose. When the glucose culture had reached an optical density at 663 nm and 1 cm light path (OD_{663}) of 1.0, cells from this culture were used to inoculate a fresh glucose culture to an OD_{663} of 0.03. This procedure was repeated three times. Cells precultured in this way and grown to an OD_{663} of 1.0 were washed once with mineral medium without carbon source and subsequently transferred into a medium containing 0.5% methanol.

A mutant of *Hansenula polymorpha* CBS 4732 lacking alcohol oxidase activity was kindly supplied by Dr. L. Eggeling KFA, Jülich, FRG.

Preparation of Spheroplasts

Spheroplasts were prepared by treatment of suspensions of whole cells with "Zymolyase" (Kitamura et al., 1971) for 5–15 min at 37° C according to the procedure of Osumi et al. (1975b).

Enzyme Assays

All enzyme assays were performed at 37° C. The preparation of cell-free extracts and the estimation of alcohol oxidase activity were as described previously (Van Dijken et al., 1976).

Catalase was assayed by the spectrophotometric method of Lück (1963), and activity is expressed as ΔE_{240}/min × mg protein.

Activities of several oxidases were determined with an oxygen electrode by the method described by Van Dijken et al. (1976) for alcohol oxidase. Urate oxidase was assayed in 50 mM borate buffer, pH 8.5, using 1 mM Na-urate as the substrate. Hydroxy acid oxidase was assayed in 50 mM phosphate buffer pH 7.5, using 150 mM D,L-Na-lactate as the substrate. Prior to the assay of D-amino acid oxidase, the cell-free extract was incubated with 1 mM FAD for 30 min at room temperature. D-amino acid oxidase activity was then determined in 0.1 M pyrophosphate buffer pH 8.2, using 20 mM D-alanine or 20 mM D-methionine as the substrate. Oxidase activities were expressed as nmol O_2 consumed/min × mg protein^{-1}.

Protein was determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

Cytochemical Staining

Cytochemical staining procedures for the detection and localization of catalase and oxidase activities were performed as described previously (Veenhuis et al., 1976).

Freeze-Etching

Cells were incubated for 10 min in 15% (w/v) glycerol, frozen in FREON and freeze-fractured in a Balzer's freeze-etch unit, according to the method described by Moor (1964).

Fixation and Post-Fixation

Whole cells were fixed with 1.5% $KMnO_4$ for 20 min at room temperature. Spheroplasts were prefixed for 30 min in 6% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 at 0° C and postfixed for 45 min in a solution of $2^1/_2$% $K_2Cr_2O_7$ and 1% OsO_4 in 0.1 M cacodylate buffer pH 7.2.

After dehydration in a graded alcohol series the cells were embedded in Epon 812. Ultrathin sections were cut with a diamond knife and examined in a Philips EM 300 or EM 201.

Quantitative Analysis of Thin Sections

The average number of peroxisomes was estimated by random counting of cell profiles in thin sections (Veenhuis et al., 1978). The surface area of the endoplasmatic reticulum and the volume density of peroxisomes, mitochondria, nucleus and vacuole in the cytoplasm was estimated with the point counting technique according to Weibel and Bolender (1976). Students' *t*-test was used for statistical analysis.

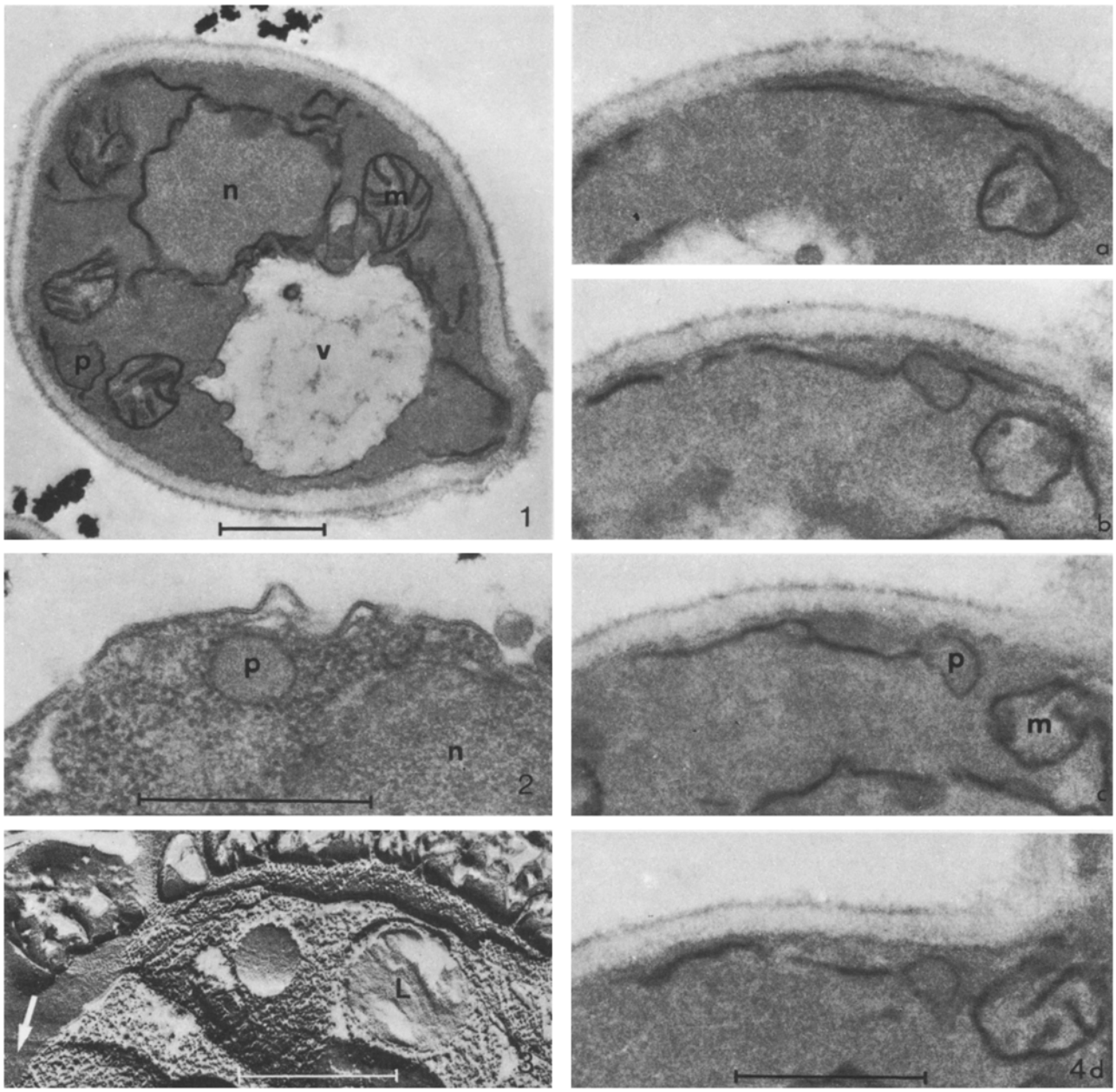
Results and Discussion

Characterization of Peroxisomes in Glucose-Grown Cells of H. polymorpha

Hansenula polymorpha, grown in batch culture in media supplemented with glucose, contained peroxisomes in all stages of growth. Serial sections revealed that during exponential growth the cells generally contained a single peroxisome, always located in close proximity to the cell wall and irregular of shape, with dimensions of 0.1–0.2 µm (Fig. 1). The matrix of these peroxisomes never showed a crystalline core (Fig. 2). The surrounding membrane, about 70 Å in width after $KMnO_4$-fixation showed, after freeze-etching of the cells, a smooth appearance (Fig. 3) identical to that of the membranes of peroxisomes in methanol-grown cells (Van Dijken et al., 1975a). The organelles were invariably closely associated with one or more strands of endoplasmic reticulum, although a direct continuity between the surrounding membrane and ER-membranes was never observed (Fig. 4a–d).

Catalase activity in these peroxisomes was demonstrated after incubation of glutaraldehyde-prefixed cells or spheroplasts with DAB and H_2O_2 (Fig. 5). The presence of activities of various oxidases was tested in spheroplasts, prefixed with glutaraldehyde, using the cerium-technique in the presence of aminotriazole in order to inhibit catalase activity (Veenhuis et al., 1978). Incubations with D-alanine (Fig. 5), Na-urate or D,L-Na-lactate showed positively stained peroxisomes, indicating the presence of D-amino acid oxidase, urate oxidase and hydroxy acid oxidase in these organelles. Activities of these enzymes were also detected in cell-free extracts of whole cells (Table 1). Incubations with $CeCl_3$ and methanol invariably showed negative results, indicating that alcohol oxidase was not present. This result was confirmed by biochemical analysis, since in cell-free extracts alcohol oxidase activity was not detected (Table 1).

Major differences were found between peroxisomes in cells from the exponential and the stationary growth phase, due to the fact that the peroxisomes changed during the late exponential and early stationary growth



Electron micrographs. Abbreviations: *L* lipoid droplet; *m* mitochondrion; *n* nucleus; *p* peroxisome; *v* vacuole. Cells were fixed/postfixed with $KMnO_4$ unless mentioned otherwise. The marker represent 0.5 μm

Figs. 1—8. Electron micrographs of cells, grown on 0.25% glucose. Figs. 1–5 were taken of cells from the exponential growth phase (OD_{663} = 1.0)

Fig. 1. Survey to illustrate the overall cell morphology

Fig. 2. Detail of a spheroplast, fixed with glutaraldehyde-OsO_4/$K_2Cr_2O_7$, indicating the absence of a crystalloid in the peroxisomal matrix

Fig. 3. Detail of a freeze-fractured cell to illustrate the smooth surface of the peroxisomal membrane. The arrow represents the direction of shadowing

Fig. 4a—d. Serial sections to demonstrate the close association of the peroxisome and the endoplasmic reticulum

phase. The organelles became more cubic in form, the diameter increased from approximately 0.2 μm to approximately 0.6–1.5 μm and a crystalline nucleus developed in the matrix of the peroxisomes (Fig. 8). In the stationary growth phase completely crystalline peroxisomes were observed (Fig. 6). This difference in ultrastructure was related to a difference in the enzymic content of the peroxisomes. Biochemical experiments

Table 1. Activities of peroxisomal enzymes in cell-free extracts of *Hansenula polymorpha*, grown in media with 0.25% glucose. Cells were harvested from the exponential growth phase ($OD_{663} = 1.0$) and the early stationary growth phase ($OD_{663} = 2.8$). Catalase activity is expressed as ΔE_{240}/min × mg protein, oxidase activities as nmol O_2 consumed/min × mg protein

Specific activities of					
OD_{663}	Catalase	Alcohol oxidase	Urate oxidase	D-Amino acid oxidase	L-α hydroxy acid oxidase
1.0	10	0	4	56	14
2.8	109	95	37	53	18

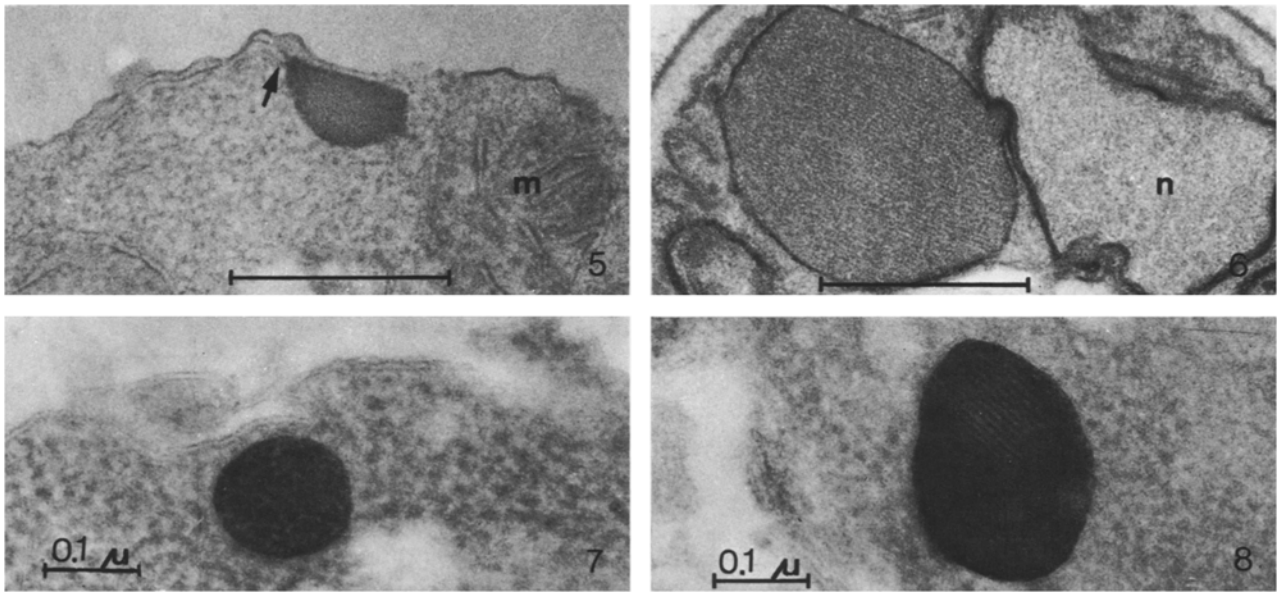


Fig. 5. Detail of a spheroplast to demonstrate catalase activity in the peroxisome after incubation with DAB and H_2O_2. Note the association with the endoplasmic reticulum (*arrow*). Fixation $OsO_4/K_2Cr_2O_7$

Fig. 6. Detail of a cell from the late stationary growth phase, showing a large peroxisome with an almost completely crystalline matrix

Fig. 7. Detail of a spheroplast from a cell from the mid-exponential growth phase, incubated with $CeCl_3$ and D-alanine to demonstrate D-amino acid oxidase activity in the peroxisome ($OsO_4/K_2Cr_2O_7$). Note the absence of a crystalloid in the organelle

Fig. 8. Detail of a spheroplast from a cell from the early stationary growth phase, incubated with $CeCl_3$ and methanol to demonstrate alcohol oxidase activity in the peroxisome ($OsO_4/K_2Cr_2O_7$). Note the crystalline inclusion, present in the peroxisomal matrix

showed that in addition to the enzymes already described, alcohol oxidase was present in cells from the early stationary growth phase (Table 1). The synthesis of this enzyme is known to be regulated by catabolite repression (Sahm and Wagner, 1973). Eggeling and Sahm (1978) have demonstrated derepression of the synthesis of alcohol oxidase in *H. polymorpha* during the later stages of the stationary phase of glucose-grown batch cultures. Our observations, however, suggest that this enzyme is already synthesized in the late exponential and early stationary growth phase. Cytochemical staining experiments, using $CeCl_3$ and methanol as a substrate, showed that alcohol oxidase activity is present in the peroxisomes, both in the crystalloid and in the non-crystalline matrix (Fig. 8). Serial sections revealed that the cytochemical detection of alcohol oxidase activity was restricted to those organelles which contained a crystalline matrix. Apart from the synthesis of alcohol oxidase, the biochemical experiments also indicated an increase in the catalase and urate oxidase activity in cells in the early stationary growth phase (Table 1). The possible relation between the increase in enzyme activities described above and the development of a crystalloid in the peroxisomal matrix was investigated using alcohol oxidase-negative mutants of *H. polymorpha*. Electron microscopical observations revealed that peroxisomes were present in glucose-grown cells both in the exponential and the stationary growth phase. However, crystalline inclusions were not observed although catalase and urate

oxidase were present to the same extent as in wild type cells.

The biogenesis of peroxisomes in glucose-grown cells remains unclear. Several authors have suggested that peroxisomes might be derived from the endoplasmic reticulum (de Duve, 1973; Gruber et al., 1973; Hruban and Rechcigl, 1969; Masters and Holmes, 1977). With regards to the distinct association of strands of ER and peroxisomes in glucose-grown cells of *H. polymorpha*, such a de novo synthesis of these organelles cannot be excluded. In budding cells we have made several observations which indicated that division of peroxisomes in these cells followed by a subsequent transfer of the newly formed organelle into the developing bud may be an alternative mechanism. Such a mechanism has been described for the multiplication of peroxisomes in drug-stimulated rat liver (Legg and Wood, 1970) and in *n*-alkane and methanol-grown yeast cells (Osumi et al., 1975a; Veenhuis et al., 1978).

The Origin and Development of Large Peroxisomes after Transfer of Glucose-Grown Cells into Methanol

The transfer experiments were performed with mid-exponential glucose-grown cells (OD_{663} = 1.0), cultivated as described above. Cells, grown in this way, did not contain any alcohol oxidase activity, as was established by both biochemical and cytochemical techniques. After the transfer of these cells into a medium containing 0.5% methanol, growth generally started after a lag of 4–6 h (Fig. 9A). Synthesis of alcohol oxidase and catalase started 2 h after the transfer and the activity of both enzymes had increased over 10-fold after 6 h of cultivation (Fig. 9B). In the same time interval activities of hydroxy acid oxidase, D-amino acid oxidase and urate oxidase remained approximately constant.

Electron microscopical observations on thin sections of $KMnO_4$-fixed cells revealed that no visible changes had occurred in the cells within the first 2 h after the change over to methanol (Fig. 10). After this period the size of the peroxisomes originally present gradually increased (Fig. 11). Two subsequent stages of growth of peroxisomes are shown in Figs. 12 and 13. The organelles were still located close to the lateral wall and had an irregular shape. During all stages of development associations with the endoplasmic reticulum remained evident. In addition to growth of a single organelle, occasionally more organelles generally 2 or 3 – were found in the early stages of their development (Fig. 14). Sections of glutaraldehyde – $OsO_4/K_2Cr_2O_7$ – fixed spheroplasts revealed the presence of a very small crystalline core in the growing peroxisomes 2 h after the transfer (Fig. 16). Subsequent growth of the peroxisomes was found to be correlated

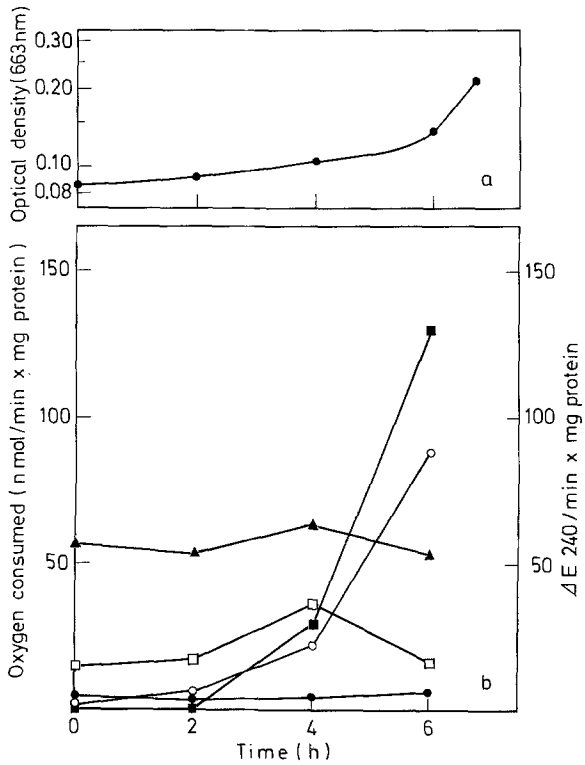
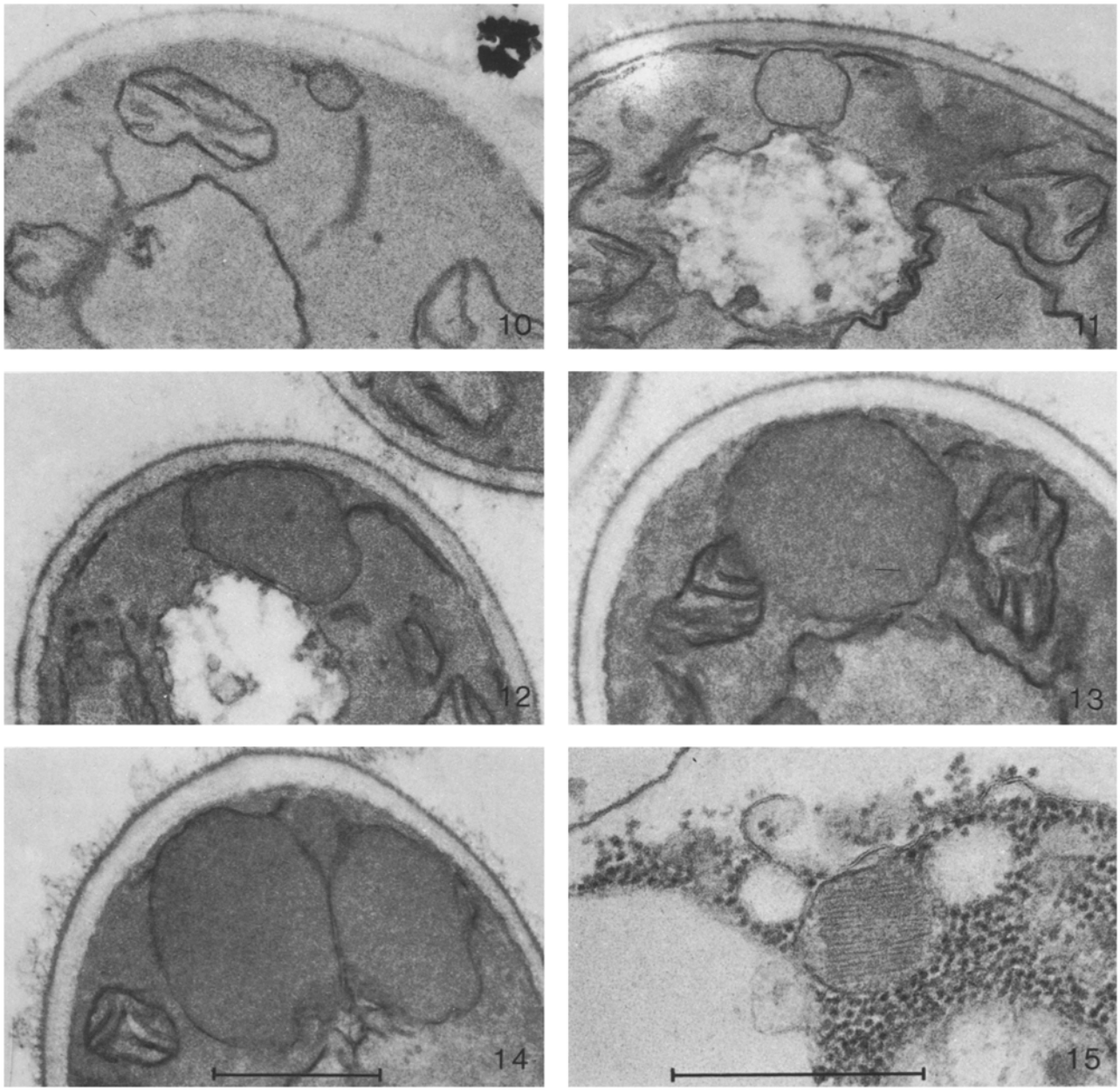


Fig. 9a and b. Growth and enzyme activities of *Hansenula polymorpha* in batch cultures supplemented with 0.5% methanol. Cultures were inoculated with cells from the mid-exponential growth phase of a culture growing on 0.25% glucose (OD_{663} = 1.0). **a** Growth (●), expressed as optical density at 663 nm. **b** Activity of catalase (○), alcohol oxidase (■), D-amino acid oxidase (▲), L-α hydroxy acid oxidase (□) and urate oxidase (●). Catalase activity expressed as ΔE_{240}/min × mg protein, oxidase activities as nmol O_2/min × mg protein

with a simultaneous growth of the crystalloid (Figs. 15 and 17). However, completely crystalline organelles were not observed in the cells at this stage of the culture.

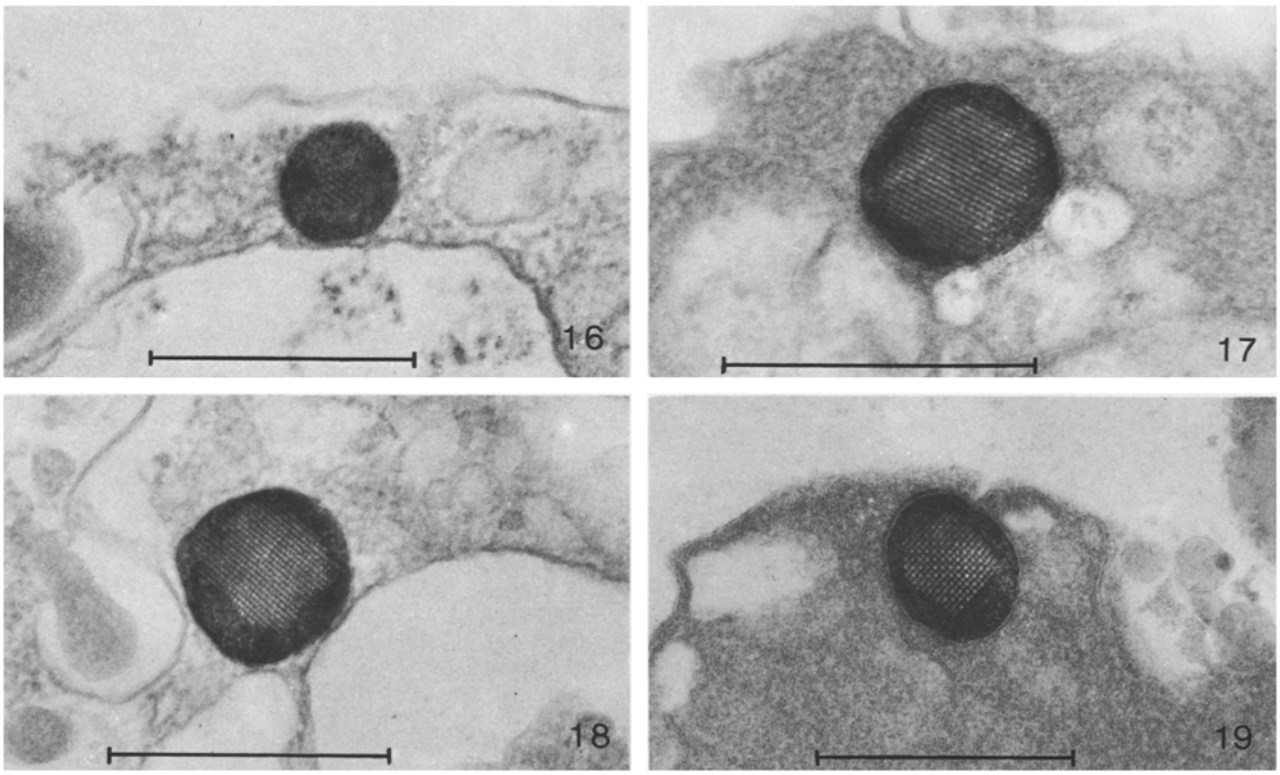
Cytochemical staining experiments, performed on glutaraldehyde-fixed spheroplasts, showed that alcohol oxidase was already present in the peroxisomes of some cells 2 h after the transfer (Fig. 16). It was observed, as described above for the peroxisomes in stationary phase cells grown on glucose, that the detection of alcohol oxidase activity was strictly dependent on the presence of a crystalline core in the organelles. However, the non-crystalline part of the organelle always stained more intensely than the crystalloid (Fig. 17), a pattern generally observed for peroxisomes in methanol-grown *H. polymorpha* (Veenhuis et al., 1978). The other enzymes investigated, namely hydroxy acid oxidase, D-amino acid oxidase, urate oxidase and catalase all were located in the peroxisomes and demonstrated a staining pattern similar to that of alcohol oxidase (Figs. 18 and 19). The development of peroxisomes in the lag phase after the transfer from



Figs. 10—14. Micrographs, printed at the same magnification to illustrate growth and development of peroxisomes in cells, transferred from glucose-into methanol-containing media. **Fig. 10** shows a detail of a cell, grown on glucose ($OD_{663} = 1.0$), **Fig. 11** 2 h, **Fig. 12** 4 h and **Figs. 13** and **14** 6 h after transfer of the cells into methanol medium. In all micrographs associations of the peroxisome with strands of endoplasmic reticulum are evident

Fig. 15. illustrates the presence of a crystalloid in the matrix of a peroxisome, 4 h after transfer from glucose into methanol medium ($OsO_4/K_2Cr_2O_7$)

glucose to methanol media and during the subsequent first hours of growth was also followed by morphometrical techniques. Six hours after the transfer the number of peroxisomes had increased by a factor of 10 and the volume density of these organelles by a factor of 70 (Table 2). These data support the observation that apart from an increase in volume density caused by an increase in number, the individual organelles had also increased in size very quickly. Morphometrical analyses were also performed on the mitochondria, the nucleus, the vacuole and the endoplasmatic reticulum. The results did not point to a significant increase or decrease in volume densities of the mitochondria and the nucleus, or the surface area of the ER (Table 2). However, the volume density of the vacuole increased by a factor of 2 after 6 h of cultivation in methanol.



Figs. 16—19. Illustrations of cytochemical staining experiments on glutaraldehyde-fixed spheroplasts, incubated with $CeCl_3$ and different substrates. **Figs. 16** and **17** demonstrate the presence of alcohol oxidase activity in peroxisomes of cells, 2 h and 6 h after transfer of the cells into methanol medium. Note the presence of the small crystalloid, present in the peroxisomal matrix 2 h after transfer (**Fig. 16**). **Fig. 18** illustrates D-amino acid oxidase activity after incubation with D-alanine and $CeCl_3$, **Fig. 19** hydroxy acid oxidase activity after incubation with D-L-lactate and $CeCl_3$, both in peroxisomes of cells 6 h after transfer into methanol medium ($OsO_4/K_2Cr_2O_7$)

Table 2. Number and volume density of peroxisomes, nucleus, vacuole and mitochondria and the surface area of endoplasmic reticulum (ER) in exponentially growing glucose cells ($t = 0$) and 6 h after their transfer into methanol-containing media ($t = 6$). The number of peroxisomes is given as average number per section. Volume densities are expressed as percentage of the cytoplasmic volume, the surface area of ER as $\mu m^2/\mu m^3$ cell volume. Statistical analysis of the data listed in this table showed that the differences in number of peroxisomes and in volume density of the vacuole and the peroxisomes were significant

Time after transfer	Number of peroxisomes	Surface area of ER	Volume densities of			
			Peroxisomes	Nucleus	Mitochondria	Vacuole
$t = 0$	0.04	1.13	0.09	11.5	15.3	3.5
$t = 6$	0.4	0.82	6.4	13.1	18.3	6.7

The results obtained clearly indicate that the increase in volume density of the peroxisomes after the transfer of the cells from glucose into methanol media is caused by the synthesis of alcohol oxidase and the dramatic increase in catalase activity. The development of crystalloids in the organelles was always correlated with the synthesis of alcohol oxidase in the cells. Since peroxisomes in alcohol oxidase-negative mutants of *H. Polymorpha* or *Candida biodinii* (Sahm et al., 1975) did not contain crystalline inclusions, it appears that the development of crystalloids in peroxisomes is dependent on the synthesis of alcohol oxidase. Further evidence in support of this view came from electron microscopy of crystallized alcohol oxidase which was purified from *H. polymorpha* (van Dijken et al., 1976). These studies revealed a periodicity of approximately 105 Å in the crystallized enzyme, which is identical to the periodicity of crystalloids in peroxisomes of methanol-grown *H. polymorpha* (Veenhuis et al., 1976). Several other oxidases have been observed in crystalline peroxisomes in methanol-grown cells of *H. polymorpha* (Veenhuis et al., 1976). It is suggested

that these enzymes are incorporated into growing crystalloids, which are initiated by the crystallization of alcohol oxidase.

The results described above differ from the observations of Tanaka et al. (1976) on *Kloeckera* sp. 2201. In cells of this organism 5–6 peroxisomes developed 4 h after the transfer from glucose to methanol media. Prolonged cultivation showed no additional increase in number but a subsequent increase in size of these organelles was observed. In *H. polymorpha*, the peroxisomes, originally present in the glucose-grown cells, increased in size after transfer of the cells into methanol media as a result of adaptation to the new environment. After the development of this organelle, which can now be regarded typical for methanol-grown cells, prolonged cultivation of the cells in methanol media resulted in a gradual increase in number of peroxisomes, caused by the formation of small peroxisomes from mature organelles (Veenhuis et al., 1978). The peroxisomes in budding cells originated from mature organelles in the mother cell by division and were subsequently transferred into the developing bud. De novo synthesis of peroxisomes by the formation of dense cores, followed by the formation of a limiting membrane derived from the endoplasmic reticulum as described by Tsubouchi et al. (1976) was not observed.

The mechanisms involved in peroxisomal growth, remain largely unclear. Associations with the endoplasmic reticulum apparent in both glucose and methanol-grown cells may point to a possible function of this organelle in the synthesis of the peroxisomal membrane. A role of the endoplasmic reticulum in the synthesis or transport of peroxisomal enzymes is more difficult to envisage, since we did not observe any significant increase in the surface area of this organelle in cells in which peroxisomes were rapidly growing. In this respect the situation appears to be similar to that observed in yeast grown on n-alkanes (Osumi, 1975a). As in previous studies (Veenhuis et al., 1976, 1978) activity of the "typical" peroxisomal enzymes investigated in the present work was only observed inside the peroxisomes; we never observed any activity in the cytosol. Further work on the synthesis, subsequent transport and possible activation of these enzymes inside the peroxisomes is now in progress.

Acknowledgements. We wish to thank J. Zagers for skillful assistance in preparing the figures and the electron micrographs and K. Zwart and Dr. J. P. van Dijken for stimulating discussions.

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Received August 17, 1978